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High prevalence of multiple paternity within fruits in natural populations of *Silene latifolia*, as revealed by microsatellite DNA analysis

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ABSTRACT

Multiple paternity within broods has been gathered in several animal species, and comparable data in plants would be of great importance to understand the evolution of reproductive traits in a common framework. In this study, we first isolated and characterized six microsatellite loci from the dioecious plant *Silene latifolia* (Caryophyllaceae). The polymorphism of the loci was assessed in 60 individual females from four different populations. Two of the investigated loci showed a pattern of inheritance consistent with X-linkage. These microsatellite loci were highly polymorphic and therefore useful tools for parentage analysis. We then used four of the markers to determine paternity within naturally–pollinated fruits in four European populations. This study revealed widespread multiple paternity in all populations investigated. The minimum number of fathers per fruit varied from 1 to 9, with population means ranging from 3.4 to 4.9. The number of fathers per fruit was not significantly correlated with offspring sex ratios. High prevalence of multiple paternity within fruits strongly suggest that pollen competition is likely to occur in this species. This may substantially impact male reproductive success and possibly contribute to increase female and offspring fitness, either through post-pollination selection or increased genetic diversity. Wide variation in outcrossing rates may be an overlooked aspect of plant mating systems.

INTRODUCTION

Over the past decades, evidence has accumulated that females of many animal species across phyla mate with multiple males during one reproductive period (e.g. Feldheim et al. 2004; Harshman & Clark 1998; Imhof et al. 1998, Bretman & Tregenza 2005), and that the resulting post-mating competition for fertilization has led to a variety of adaptations in both sexes (e.g. Arnqvist & Rowe 2005). In plants, by analogy, variation in outcrossing rates and pollen competition for fertilization of the ovules in a single fruit may have important evolutionary consequences. Polyandry (which we use here *sensu lato* also for plants, to describe the number of donors contributing to fertilization of the seeds within one fruit) is important for male and female fitness, interactions of the parental plants and their offspring, and gene flow (Snow & Lewis 1993). Receiving pollen from multiple donors may increase female fitness by enhancing the genetic quality or diversity of their offspring, or by hedging against sterility, genetic defects or selfish genetic elements present in partners (Haig & Bergström 1995; Schlichting et al. 1987; Bernasconi et al. 2004). The occurrence of polyandry in natural populations has direct implications for selection on floral and inflorescence traits that enhance pollen export and import in entomophilous plants, and selection on pollen competitive ability (Janzen 1977; Ellstrand 1984; Delph & Havens 1998; Barrett 2003; Wright & Meagher 2004). Maintaining floral receptivity so as to collect pollen from multiple donors, however, may also bear costs. Costs may arise for example through enhanced risk of contracting sexually transmitted fungal diseases (Kaltz & Shykoff 2001), time and energy for maintenance of receptive floral structures, increased risk of attracting specialized herbivores, such as seed predators, that may respond to floral display (Wolfe 2004). Therefore, floral traits may evolve in females to ensure optimal levels of donor diversity, while balancing the benefits and costs of increased floral display or prolonged receptivity, and in males to maximize pollen export and siring success (Barrett 2003).

Early studies using allozymes generally indicated multiple within–fruit paternity (reviewed in Bernasconi 2003; Mitchell et al. 2005) and multiple mates for both males and females (e.g. Meagher 1986). Highly polymorphic molecular markers, such as microsatellite DNA enhance the likelihood of detecting multiple paternity and thus also potential pollen competition in natural populations because of their higher resolution, and because they are expected to be neutral with respect to gametophytic selection or pollinator preference. When phenotypic markers are used to assign paternity, a potential problem arises if they are not neutral, that is, if they are associated with differential paternity success (Stanton et al. 1986). This may also occur for different alleles at electrophoretic loci, if they affect paternity either directly, indirectly via linked loci, or through differences in post-zygotic seed abortion. Although this may not be a general problem, in at least one species hand-pollination experiments revealed differential siring success of pollen donors differing at the *Pgi* locus (Travers & Holtsford 2000, Travers & Mazer 2001). Despite their potential usefulness, to our knowledge only one study to date has applied microsatellite DNA to explore the frequency of within–fruit multiple paternity in plant populations (Reusch 2000).

We investigated within-fruit paternity in the white campion. *Silene latifolia* has separate sexes. Males produce many more flowers than females (Delph et al. 2002), and increasingly so during the flowering seasons (Meagher & Delph 2001; Elzinga & Bernasconi, in review), which should ensure pollen availability during most of anthesis especially in larger populations. Thus, multiple paternity is likely to occur and pollen competition may play an important role in shaping the evolution of reproductive traits. From a female perspective, collecting excess pollen from multiple donors may increase genetic variability and offspring fitness, and provide opportunity for post-pollination gamete or zygote selection. Because seeds disperse locally (McCauley 1997; Richards et al. 1999; Wright & Meagher 2004), nearby individuals are likely to be more closely related than distant individuals (M. Barluenga, J.A. Elzinga, S. Teixeira, J. Goudet and G. Bernasconi, unpublished

data). Thus, inbreeding avoidance through post-pollination selection may be particularly relevant in *S. latifolia*. Finally, polyandry may allow females to avoid fertilizations by males carrying meiotic drive alleles. Indeed, a previous study in *S. latifolia* found that males producing broods with biased sex ratios sired fewer offspring when competing against normal males, but both types of males had equal numbers of progeny in single-donor crosses (Taylor et al. 1999). This suggests a potential role for pollen competition in the avoidance of sex ratio distortion.

Here, we isolate and characterize microsatellite DNA markers and used them, both autosomal and X-linked, to estimate the number of non-maternal alleles per fruit and reconstruct paternal genotypes in four geographically separated *S. latifolia* populations from the European distribution range, in order to assess the frequency of multiple paternity in each of the study populations.

MATERIAL AND METHODS

Study species

The white campion [Silene *latifolia* (Miller) Kraus] is a diploid (2n = 24) member of the carnation family, Caryophyllaceae. It has an X/Y chromosomal sex determination system, with heterogametic males (Westergaard 1958). It is a short-lived perennial, insect-pollinated species, with fruits producing around 200 seeds. *Silene latifolia* is a model system in diverse fields, including the study of host-pathogen interactions (Alexander et al. 1996; Kaltz & Shykoff 2001), sex chromosome evolution (Guttman & Charlesworth 1998; Ironside & Filatov 2005), sexual dimorphism (Meagher 1992, 1994; Delph et al. 2002), sex ratio distortion (Taylor et al. 1999), and invasive species biology (Wolfe et al. 2004).

Field collection

In June and September 2003 we sampled leaves and fruits from 15 plants in each of four natural populations in Central Europe: Cottendart (CT, 46°58'30" N; 6°50'50" E; >1000 flowering plants), Montbrun-les- Bains (MB, 44°10' 00''N; 5°25'00'' E, ca. 500 flowering plants), Sesto Calende (SC, 45°44'08" N; 8°37'00" E, >100 flowering plants) and Village Neuf (VN, 47°36'25" N; 7°33'31" E, ca. 80 flowering plants). We sowed 20 seeds per fruit for a total of 1200 seeds (4 populations * 15 maternal parent * 20 offspring), in Petri dishes with 1 mM giberillic acid in a growing cabinet (21°C, 70% RH, 16 h day / 8 h night). We then sampled seedlings for leaf tissue.

Isolation and characterization of microsatellite markers

Total genomic DNA was extracted from leaf samples of five individuals from a natural, european *S. latifolia* population, using the cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle & Doyle 1990). An enriched library was made by ECOGENICS GmbH (Zürich, Switzerland) from size-selected genomic DNA ligated into TSPAD-linker (Tenzer et al. 1999) and SULA/B-linker (Armour et al. 1994) for GA and CA, respectively, and enriched by magnetic bead selection with biotin-labelled $(CA)_{13}$ and $(GA)_{13}$ oligonucleotide repeats (Gautschi et al. 2000). Of 384 recombinant colonies screened, 100 gave a positive signal after hybridization. Plasmids from 48 positive clones were sequenced and primers were designed for 10 microsatellite inserts. Of these, 6 were highly polymorphic. Sequences have been deposited in GenBank (accession no. DQ469337 through DQ469344).

We estimated the level of genetic variability by genotyping 60 field-collected females, 15 from each of the four populations from central Europe (Village-Neuf/F, Montbrun-les-Bains/F, Cottendart/CH

and Sesto Calende/I; see under *Field collection*). Number of alleles, observed and expected heterozygosity were computed using the program GENETIX 4.05 (Belkhir et al. 2004).

Microsatellite genotyping

We extracted genomic DNA to use as a template for polymerase chain reaction (PCR) from the leaves of single maternal parents using the CTAB method (Doyle & Doyle 1990). We extracted DNA from leaf material of offspring using a commercial extraction kit (Macherey-Nagel Nucleospin Plant Kit). The extracted DNA was run in 0.8% agarose gels to estimate DNA concentration so that a final concentration of 10 ng/µL could be obtained by dilution. We performed the PCR with four fluorescent-labelled primers (Sl6, Sl8, Sl14 and Sl15). Variability among individuals was analysed by polymerase chain reaction (PCR) in 10 µL reaction volumes containing 10 ng of genomic DNA, 1x Qiagen Multiplex PCR master Mix and 2 µM of each primer for the Multiplex-PCR. For the reactions with only one primer pair, we used the Qiagen HotStarTaq Master Mix. The PCR was performed with fluorescent labelled primers: Sl1 (FAM); Sl4 (HEX); the duplex Sl6 (FAM) and Sl8 (HEX) and the duplex Sl14 (FAM) and Sl15 (HEX). The PCR amplification was conducted in a Biometra thermocycler with the following program: 15 min. at 95°C; 30 cycles composed of 30s denaturation at 94°C; 90s at melting temperature (T_m given in Table 1) and 60s elongation at 72°C, followed by a final elongation step of 30 min. at 60°C. Fragments were separated on an ABI 3100 genetic analyser (Applied Biosystems) with the internal size standard Genescan 350. Fragments were separated on an ABI 3100 genetic analyser (Applied Biosystems) with the internal size standard Genescan 350. We analysed fragments using Genemapper (Applied Biosystems). Due to failure of germination (up to 7%, see also Jolivet & Bernasconi 2007a) or amplification via PCR of some of the offspring, the final sample for paternity analysis included 919 offspring.

Paternity analysis

We analysed paternity within field-collected fruits using four of the six markers, since thanks to high polymorphism (see Results) this was sufficient to address prevalence of multiple paternity. We scored maternal and offspring genotypes to identify maternal alleles in the offspring. The remaining non-maternal alleles present in each fruit were used as an estimate of the number of paternal alleles. This was repeated for each marker locus. We considered homozygote offspring as being issued from a father having the same allele as the mother. This is appropriate because *S. latifolia* has separate sexes and progeny can thus only arise through cross-fertilization.

Taking advantage of the fact that two of our four markers were X-linked (see Results), we followed the methodology of Walker et al. (2005) for parental genotype reconstruction. To estimate the number of fathers we first examined the genotype at the two X-linked markers and then used the two autosomal markers to further resolve paternity using multilocus genotype reconstruction, i.e. we estimated the minimum number of multilocus genotypes necessary to account for genetic variation in the offspring. The paternal allele at the X-linked markers is inherited only to daughters (and absent in sons). For daughters, each distinct paternal haplotype at one X-linked marker was interpreted as one distinct father. If a single paternal haplotype at the X-linked markers was associated with more than two non-maternal alleles at the autosomal markers, we inferred the presence of more than one male genotype. Sons are hemizygous at the X-linked markers and therefore for sons we inferred paternal genotypes only based on autosomal loci. For offspring within one fruit, we interpreted the genotypes of sons at the autosomal markers by grouping them according to the genotypes inferred for the daughters and only if new alleles appeared in the subset of male offspring, we considered this as indicating a further sire.

We estimated the paternity shares obtained by the different fathers in each fruit, by calculating an index K_E for the effective number of fathers as $K_E = 1/\sum(p_i)^2$, where p_i is the proportion of offspring each of the $i = 1 \ldots K$ pollen donors sired per fruit (Bernasconi 2003). This index K_E is maximized (and then equals the number of donors, K) when all fathers sire the same number of offspring, as expected if paternity is random. To assess paternity shares, we only used data from daughters and the X-linked markers, since this provides the most accurate estimate based on the paternal X-chromosomal haplotype.

We tested whether K and K_E significantly deviated from the null hypothesis of monandry, using one sample t-tests against the null hypothesis *"K, K_E = 1"* for each population separately. We tested whether K_E values differed significantly among populations using univariate ANOVA with population as a random factor.

In the paternity analysis we excluded some families that showed a pattern of null alleles (3 families out of 60 were excluded: 2 from SC and 1 from CT, see also Jolivet & Bernasconi 2007a).

RESULTS

Marker loci polymorphism and sex-linked inheritance of Sl14 and Sl15

The six loci were highly polymorphic, with 25 to 43 alleles per locus; H_O ranged from 0.23 to 1.0 and H_E from 0.86 to 0.97. Some loci (Sl1, Sl4 and Sl8) showed lower observed than expected heterozygosity (Table 1). This may be due to limited sample size, spatial genetic structuring due to limited seed dispersal, or the existence of null alleles. When considering the offspring sample (n =

919), in four populations the most common allele at each of the loci Sl6, Sl8, Sl14 and Sl15 had maximum frequencies of 0.22, 0.31, 0.27, 0.23, respectively (Table 2). This confirms the high polymorphism of the markers.

At locus Sl14 and Sl15, females generally had two alleles, while males were hemizygous (Table 3), while the same males and females generally had two alleles at locus Sl6 and Sl8 (Table 3a). Separate controlled pollinations and sex determination of flowering offspring revealed that males transmitted their allele at Sl14 and Sl15 exclusively to daughters, while female alleles were found in both daughters and sons (Table 3b). This strongly suggests that locus Sl14 and Sl15 show X-linked inheritance. This pattern is consistent in geographically separate populations from the European native range of the species. Significant linkage disequilibrium was detected between Sl1, Sl14 and Sl15 (Black & Krafsur 1985 procedure, with permutation tests).

We additionally verified in one population (VN) that the estimated sex ratio of offspring (scored by the absence (in sons) vs. presence (in daughters) of a second allele at both the X-linked loci) corresponded to observed sex by growing the offspring until they started flowering. There were only 9 mismatches out of 158 offspring; i.e. the sex assignment based on markers was correct in 94% of cases; the 6% mismatches were homozygous females. Thus, although sex determination would be more secure when using Y-linked markers, we obtained a very high certainty also with X-linked markers.

Offspring sex ratios (percentage females within seed families; mean ± s.d. n=20 offspring/fruit, estimated from Sl14 and Sl15, i.e. by considering as males individuals bearing maternal alleles only at both Sl14 and Sl15) were 67% ± 20%; 58% ± 18%; 79% ± 20% and 57% ± 14% in CT, MB, SC

and VN, respectively. In 15 out of 57 families (26%; by population: 3/14 in CT, 3/15 in MB, 8/13 in SC and 1/15 in VN), the offspring sex ratio was significantly biased (i.e. p-value of binomial test <0.05). In these "sex-ratio biased families", a vast majority (14/15) had an excess of daughters.

Number of fathers and paternity shares

Overall, the maximum number of paternal alleles per fruit varied from 2 to 10, with population means ranging from 3.8 to 6.1. If we take into consideration the X-linked loci, the presence of 2 or more paternal alleles is indicative of multiple paternity. By this criterion, the proportion of multi-sired fruits in each population were: CT: 100% (14/14); MB: 87% (13/15); SC: 100% (13/13); VN: 60% (9/15). These results show clearly that the vast majority of fruits had more than one inferred father, although with a lower level for the smallest population VN. Accordingly, paternal genotype reconstruction based on the two X-linked and the two autosomal loci also revealed multiple sires with a number of fathers per population ranging from 1 to 9, with population means ranging from 3.4 to 4.9 (Table 4; Fig. 2).

We estimated the number of donors with three measures: a) The number of inferred donors (K) based on both sons and daughters and four loci (two autosomal and two X-linked); b) the number of inferred donors (K) based on daughters and two X-linked loci; and c) the effective number of fathers K_E calculated from the two X-linked loci and considering only daughters.

Within all populations K and K_E were significantly larger than 1, indicating that polyandry is prevalent in all populations (one sample t-tests, Table 4). Polyandry was frequent in all study populations and populations did not differ significantly (univariate analysis of variance with population as random factor; K: $F_{3,53 = 2.03;} p = 0.12$ K_d: $F_{3,53} = 2.47$; $p = 0.072$), or differed only marginally (K_E: $F_{3,53} = 2.87$; $p = 0.045$) in the prevalence of multiple paternity. The smallest

population (VN) had the lowest values of multiple paternity, however our conservative estimate revealed that also in this population seed families were sired on average by at least 2 to 3 donors.

DISCUSSION

Analysis of paternity in plant population is fundamental for assessing male reproductive success, for understanding the evolution of floral and inflorescence traits, as well as for quantifying the opportunity for post-pollination selection (Snow & Lewis 1993; Bernasconi 2003). Post-pollination selection may be particularly relevant in plants, which cannot choose with whom they mate. However, very few studies (e.g., Ellstrand 1984; Campbell 1998; Reusch 2000) have investigated paternity within fruits in natural plant populations and to our knowledge this is the first study to apply microsatellite DNA markers to investigate within-fruit polyandry under natural conditions in an animal-pollinated plant.

Our study reveals that the vast majority of seed families in the studied populations of *S. latifolia* have more than one sire. Polyandry was highly prevalent in all four investigated populations, which originated from geographically distant locations within the European native range of the species. Population size ranged from around 80 individuals (VN) to more than a 1000 individuals (CT). In the smallest population (VN) the number of singly–sired fruits was higher than for the remaining populations, however there were only marginally significant differences in the level of within-fruit polyandry (effective number of fathers) among the study populations, with the smallest population still having significant polyandry. Although we did not have small populations in our study, a possible limitation of our results is that population size and isolation could alter the prevalence of polyandry, especially if very small populations are considered. *Silene latifolia* commonly occurs in

natural meta-populations (Elzinga et al. 2005). Small isolated sub-populations may have lower multiple paternity rates than observed in our study, because of limited number of males, female-biased sex ratios, or pollen and pollinator limitation. Indeed, polyandry was previously thought to be relatively rare in this species, at least in North American populations (Taylor et al. 1999). It would be interesting to know whether in small populations post-pollination selection favouring one donor over others would become more important, resulting in greater paternity skew (Vergnerie 2006).

Our estimate of multiple paternity clearly revealed a high prevalence of polyandry, with over 90% of the seed families being sired by multiple fathers. This estimate does not correct for the probability of different males having the same genotype. We estimated allele frequencies for a larger sample of individuals in six populations including CT, VN and SC (Jolivet & Bernasconi 2007a), but we cannot use them for calculating non-exclusion probabilities in the present study, because due to high polymorphism alleles present in the mother - offspring arrays examined here were often different from those present in the subset of individuals used for allele frequency estimation. Nevertheless, high polymorphism and the multilocus approach suggest that non-exclusion probabilities were likely to be very low. In particular, the heterozygosity values (H_E=0.86-0.97) and also the estimated frequencies of the most common alleles (Table 2) suggest that the probability that two fathers drawn at random from the same population would have the same multilocus genotype would be less than 10^{-4}.

In *S. latifolia*, multiple paternity may result from pollen from several donors being deposited during a single pollinator visit (pollen carry-over), or from multiple visits by pollinators (Barthelmess et al. 2006). It would be interesting to know whether there are traits favouring polyandry in this species,

for instance flower size, flowering display size (Barrett 2003; Mitchell et al. 2005), other components of pollinator attraction, or stigma receptivity schedules (Lankinen et al. 2006).

Enzyme electrophoresis has been used to estimate the occurrence of multiple paternity in natural populations (Ellstrand 1984; Smouse & Meagher 1984; Meagher 1986; Campbell 1998; Smouse et al. 2001). For instance, in *Raphanus sativus,* multiple paternity (with up to 4 sires per fruit) has been documented in all individuals and 85 % of all fruits in one natural population (Ellstrand 1984). High levels of multiple paternity were also found in natural populations of the hummingbird-pollinated *Ipomopsis aggregata*. Isozyme markers revealed multiple paternity in the majority of fruits, with on average 4 sires per fruit (which contained up 2- 14 seeds; Campbell 1998). Analysis of paternity for the progeny of 69 females in the dioecious *Chamaelirium luteum* (Liliaceae), revealed that variances in the number of mates differed greatly between the sexes (females: 4.9, males: 37.8; Meagher 1986). Thus, although only few studies addressed multiple paternity within fruits, they generally found relatively high levels of it in natural populations, suggesting that this may be an important but overlooked aspect of plant reproductive ecology.

In our study we cannot test whether paternity within fruits is uneven, since we do not know how much pollen and from how many different donors was received by the female plants. A recent study found significant variation in male reproductive success in *S. latifolia* using maximum likelihood analysis of allozyme variation (Wright & Meagher 2004). In the investigated population, some males obtained very high reproductive success, while most males only obtained low paternity (Wright & Meagher 2004). This is similar to results obtained in another dioecious species: in *C. luteum,* individual males made significantly unequal contributions to the progeny pool, with many males virtually not contributing to the next generation (Smouse & Meagher 1994). Variance in male reproductive success may result from random variation for instance in the timing of pollen

deposition, or from non-random factors such as differences between pollen donors in attractiveness to pollinators, or in variation in seed siring ability, post-pollination selection in the style, and interactions between pollen recipient and pollen donor genotypes (Bernasconi 2003). Clearly, the elucidation of mechanisms can only be resolved in controlled experimental manipulations.

More studies are also needed to understand the consequences of multiple-donor pollination and within-fruit genetic variability for maternal, paternal and offspring fitness. Polyandry within fruits reduces the genetic relatedness among offspring, which in some cases may enhance female and offspring fitness. For example, in both wild radish (*Raphanus sativus*; Marshall & Ellstrand 1986) and the endemic *Chochlearia bavarica* (Paschke et al. 2002), multiple paternity within fruits had a positive effect on female reproductive success by increasing seed set per fruit. Increased female fitness as a result of increased genetic variability of the offspring might be advantageous in a fluctuating environment (Schlichting et al. 1987). For maternal fitness, polyandry may allow the sampling of an optimal number of males, or of particularly compatible males and provide opportunity for post-pollination selection (Delph & Havens 1998; Bernasconi et al. 2004). In *S. latifolia*, Taylor et al. (1999) suggested that males that produce offspring with female-biased sex ratios may be disfavoured at pollen competition. This may predict an association between the degree of polyandry and evenness of offspring sex ratio. We found over one fourth of the families in this study to have significantly biased offspring sex ratios (in all but one case an excess of daughters), but no evidence that sex-ratio biased families differed in the number of donors compared to families without sex ratio bias (data not shown). It would be interesting to conduct experimental pollinations varying donor numbers to investigate experimentally whether increasing levels of polyandry beyond double-male pollinations leads to more progeny of higher fitness and also more even offspring sex ratios.

In conclusion, within-fruit multiple paternity frequently occurs in natural populations of *S. latifolia* within its native range, at least in relatively large populations (> 80 flowering individuals) such as those investigated. Given this high frequency of multiple paternity in natural *S. latifolia* populations, it is now relevant to address questions such as what mechanisms control paternity shares and what are the consequences for male and female fitness in *S. latifolia*. More generally, this study reveals that multiple paternity within fruits is frequent, suggesting that it may have potentially important consequences for male reproductive success and genetic diversity within seed families. Although scarcely studied, polyandry within fruits could have tremendous effects on mating strategies and on the selective pressure shaping reproductive traits in flowering plants (Armbruster et al. 2002). Variation in outcrossing rates and competition among pollen donors may be an underappreciated evolutionary force in plants, and the use of molecular markers in other species may help to elucidate their importance. The developed microsatellite DNA loci should be particularly useful for population genetic studies, and add to other microsatellite markers described for the genus *Silene* (Juillet et al 2003, Tero & Schlötterer 2005). Sex-linked markers should prove particularly useful in parentage analysis (Avise et al. 2004; Walker et al. 2005), sex determination before flowering, and in the analysis of sex-ratio variation in natural populations (Taylor 1999).

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LEGENDS TO FIGURES AND TABLES

Figure 1 Pie charts for the distribution of the number of inferred fathers (*K*) in field-collected seed families from four *Silene latifolia* populations. Labels inside the slices indicate number of fathers; the size of slices is proportional to the number of seed families. Grey: families with one inferred father.

Table 1. Characterization of six polymorphic microsatellite loci isolated from the white campion *Silene latifolia*: locus name, primer sequences (F: forward primer, R: reverse primer), melting temperature (T_m), repeat motif from sequenced clone, number of alleles (N_A), observed allele size range (bp), observed (H_O) and expected (H_E) heterozygosity. Sixty individual females were analyzed for each locus (15 from each of four natural populations).

Table 2. Number of alleles per locus (=A) and frequency of the most common allele (=*f*) at four microsatellite DNA loci (Sl6, Sl8: autosomal; Sl14, Sl15: X-linked) in the progeny (n=919), from 60 seed families (15 in each of four populations of *Silene latifolia*).

Table 3. Analysis of sex-linked inheritance of loci Sl14 and Sl15 in *Silene latifolia*: *(a)* offspring from one representative open-pollinated fruit from Village-Neuf/F (maternal genotype known from leaf tissue, paternal alleles inferred from progeny genotype), and *(b)* offspring from one representative fruit arisen through controlled pollination using plants from Millingerwaard/NL (genotype of all parents known from leaf tissue; population characteristics described in Jolivet and Bernasconi, 2007a, 2007b). The loci Sl14 and Sl15 are hemizygous in male offspring, while female offspring generally display two alleles. By contrast, the same males have generally two alleles at Sl6 and Sl8 (a). Paternal alleles are exclusively inherited to daughters and absent in sons *(b)*.

Table 4. Analysis of multiple paternity within fruits in four geographically separated natural populations of *Silene latifolia* from the European native range. *K*= number of inferred donors per fruit based on all offspring and all loci; K_d= number of inferred donors based on daughters and the two X-linked markers; K_E daughters= effective number of donors (see Methods) calculated based on daughters and the two X-linked markers; *N offspring*= number of offspring scored; *N*= number of fruits (each from a different maternal plant); *t*= one sample t-test for the null hypothesis of single donor paternity (*K* or K_E =1); p= two-sided error probability.

1 Table 1.

Locus	Primer sequence (5'-3')	T_m (°C)	Repeat motif	N_A	Allele size	H_O	H_E	Accession no.
Sl 1	F: CGTCGACTTAACCAACTAAATGC	60	$(CA)_{31}$	43	156 - 272	0.67-0.80	0.94-0.97	DQ469337
	R: ATGCGTAGCTAAATTTCCCTTG							
Sl 4	F: CCAACCCTTTATTTATTACCCTTC	50	$(CT)_{19}CC(CT)_3T(CT)_2$	33	116 - 208	0.23-0.57	0.92-0.96	DQ469338
	R: TTGTGAAAGTATTTCAGGTTTAAAAG							
Sl 6	F: GCGTTGGGTGAAGACGTATG	60	$(GT)_{40}$	29	112 - 266	0.69-1.00	0.90-0.95	DQ469339
	R: AGCCCTTGACACTAACACCAAG							
Sl 8	F: GAAGGGTTTTTGGGGTTTAATG	60	$(GA)_{37}$	25	110 - 238	0.33-0.67	0.86-0.93	DQ469340
	R: CACTCATCATTGCCCTTGTTC							
Sl 14	F: TTGGAGTCGAGATTGGAGTAGG	60	$(GT)_{41}$	41	158 - 254	0.64-0.93	0.94-0.96	DQ469343
	R: TGAGTTGAAAATAATAAGAGGGTCAG							
Sl 15	F: AGTTATGGAGGATGAGGAGTCG	60	$(GT)_{46}$	40	110 - 208	0.80-0.93	0.91-0.97	DQ469344
	R: TAAGTCACAACCGAACAAATGC							

Table 2.

Locus	CT		MB		SC		VN	
	A	*f*	A	*f*	A	*f*	A	*f*
Sl6	50	0.06	37	0.14	22	0.17	32	0.22
Sl8	33	0.23	21	0.31	15	0.26	16	0.18
Sl14	25	0.27	26	0.15	17	0.12	28	0.12
Sl15	32	0.16	31	0.15	14	0.23	23	0.15

Table 3 a)

	Sl 14		Sl 15		Sl 6		Sl 8	
Offspring Gender*	Maternal alleles	Paternal alleles	Maternal alleles	Paternal alleles	Maternal alleles	Paternal alleles	Maternal alleles	Paternal alleles
	220 238		110 190		126 182		126 132	
F	220 or 238	220 or 238	190	148	182	182	132	168
F	220	156	190	116	182	240	126	110
F	238	238	110	148	126	136	126	118
F	220	220	190	134	126	240	132	118
F	238	238	110	148	182	240	132	132
F	238	238	110	148	126	126	132	132
F	220	234	190	148	182	240	126	110
F	238	234	110	134	126	240	126	118
M	238		110		126 or 182	126 or 182	132	188
M	220		190		126	240	132	110
M	238		110		126	240	132	118
M	238		110		182	240	132	188
M	238		110		126	126	132	188
U	220	156	190	116	126	152	132	110
U	220	220	190	134	126 or 182	126 or 182	126	118
U	238	238	110	110	126 or 182	126 or 182	132	132
U	238	238	110	110	126	240	126	168
U	220	220	190	190	182	240	132	132

*Observed gender: F=female; M=male; U=not determined (offspring did not flower)

Table 3 b)

	Sl 14			Sl 15		
Offspring gender*	Genotype			Genotype		
	Mother	Sire 1	Sire 2	Mother	Sire 1	Sire 2
	174 190	194	164	164 184	174	118
F	174		164	164		118
M	174			164		
F	190		164	164		118
M	174			164		
F	190		164	164		118
F	174	194		164	174	
F	174	194		164	174	
M	190			184		
F	190		164	184		118
F	190	194		184	174	
F	174	194		164	174	
F	174		164	164		118
F	174		164	164		118
F	174		164	164		118
F	174		164	164		118
F	190	194		184	174	
F	174		164	164		118

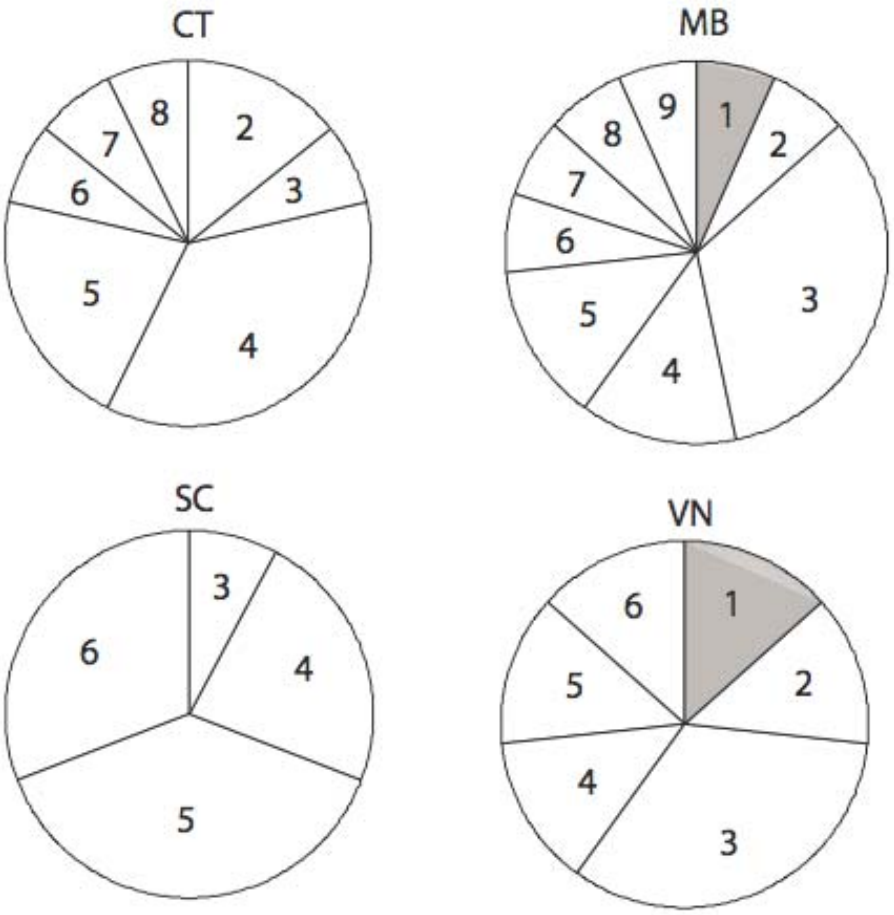
*Observed gender: F=female; M=male

1 Table 4

	N	Mean	s.d.	t	p
CT (Switzerland)					
K	14	4.50	1.70	7.71	<0.01
K_d	14	3.93	1.60	6.89	<0.01
K_E daughters	14	2.96	1.15	6.36	<0.01
N offspring	14	13.86	3.61		
MB (France)					
K	15	4.40	2.26	5.82	<0.01
K_d	15	3.93	2.25	5.05	<0.01
K_E daughters	15	2.76	1.78	3.82	<0.01
N offspring	15	16.93	2.52		
SC (Italy)					
K	13	4.92	0.95	14.83	<0.01
K_d	13	4.62	0.87	14.99	<0.01
K_E daughters	13	3.35	0.65	13.08	<0.01
N offspring	13	16.69	1.84		
VN (France)					
K	15	3.40	1.59	5.83	<0.01
K_d	15	2.93	1.53	4.88	<0.01
K_E daughters	15	2.06	0.85	4.83	<0.01
N offspring	15	15.67	2.97		

2

Figure 1



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